



Europäisches Patentamt
European Patent Office
Office européen des brevets



⑪ Publication number : **0 606 804 A2**

⑫

EUROPEAN PATENT APPLICATION

⑰ Application number : **93403185.7**

⑤① Int. Cl.⁵ : **G01N 33/48, C07K 7/08,
G01N 15/14, G01N 33/68**

⑰ Date of filing : **27.12.93**

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

⑳ Priority : **30.12.92 CA 2086453**

④③ Date of publication of application :
20.07.94 Bulletin 94/29

⑧④ Designated Contracting States :
BE CH DE FR GB LI SE

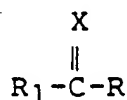
⑦① Applicant : **McGILL UNIVERSITY**
845 Sherbrooke Street West
Montreal, Quebec H3A 1B1 (CA)

⑦② Inventor : **Beaudet, Alain**
39 Surrey Drive,
Town of Mount Royal
Quebec H3P 1B2 (CA)
Inventor : **Faure, Marie-Pierre**
2286 rue Frenette
Saint-Laurent H4R 1M2 (CA)
Inventor : **Gaudreau, Pierrette**
9123 Rivard Blvd
Brossard, Quebec J4X 1V5 (CA)

⑦④ Representative : **Peaucelle, Chantal et al**
Cabinet Armengaud Aine
3, avenue Bugeaud
F-75116 Paris (FR)

⑤④ **Marker for neurotensin receptor.**

⑤⑦ The present invention relates to compound having the formula :



or a pharmaceutically acceptable acid salt thereof, wherein
R is a polypeptide moiety comprises an amino acid sequence :

-Y-Arg-Pro-Z-Ile-Leu (SEQ ID NO:1);

wherein Y is Arg, and

Z is Tyr or Trp ;

R₁ is a fluorophore selected from the group consisting of fluorescein, rhodamine, Blue fluorescent and Texas red ; and

X is oxygen or sulfur. The present invention also relates to a method for the labeling of neurotensin receptors on cell surface and a method to isolate neurotensin-receptor expressing cells.

EP 0 606 804 A2

BACKGROUND OF THE INVENTION

a) Field of the Invention

5 The invention relates to a selective fluorescent peptidic probe for neurotensin receptors, a method for *in vitro* labeling of neurotensin receptors on cell surface and a method to isolate neurotensin-receptor expressing cells.

b) Description of Prior Art

10

Neurotensin (NT) is a peptide of 13 amino acids (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH) isolated from the hypothalamus (Carraway, R. & Leeman, S.E., The J. of Biol. Chem., 1973, 248:6854-6861) and subsequently identified in the central nervous system (CNS) and the gastrointestinal tract of all vertebrates. Neurotensin fulfills a dual function as a neuromodulator in the nervous system and as a hormone in the gastrointestinal tract. Both modes of action imply as a first step the selective association of the neuro-

15

peptide with a specific receptor located on the plasma membrane of target cells.

Neurotensin-binding sites have been localized and characterized in various tissue preparations and in cell cultures from central and peripheral nervous systems. Pharmacological studies have demonstrated the existence of two populations of neurotensin binding sites : a low affinity and high capacity binding component (NTR1), from which neurotensin binding is displaced by the potent histamine antagonist, levocabastine ; and a high affinity and low capacity binding components (NTR2) corresponding to the physiologically relevant form of the receptor. Both rat and human NTR2 components have been cloned and sequenced (Tanaka, Masu, and Nakanishi, Neuron, 1990, 4:947-854). Some of the biochemical events mediating neurotensin signal transduction have also been characterized.

20

In recent years, fluoro-labeled probes have been developed for localizing peptide, cytokine, drug, and hormone receptors at the cellular level in tissue and cell cultures (Hazum E., Chang K-J, & Cuatrecasas P., Proc. of Natl. Acad. Sciences USA, 1979, 77:3038-3041). This concept, which involves labeling purified molecules covalently with fluorochromes such as fluorescein , has permitted the characterization of the kinetics, the distribution and the ultimate fate of many ligands in living cells (Taylor D.L. & Wang YuLi, Nature, 1980, 284:405-410).

30

It would be highly desirable to be provided with a non-toxic highly sensitive tool for biochemical, pharmacological and anatomical studies of the neurotensin receptor in both brain and peripheral tissues.

All markers for neurotensin receptors derived to date are of radioactive nature and hence have a limited half-life. In addition, radioactive probes for neurotensin receptors are costly and provide only static information on underlying biological processes.

35

Further, it would be highly desirable to be provided with a marker for neurotensin receptor which would allow for the isolation of neurotensin-receptor expressing cells. This application is of particular interest in view of the demonstration of a selective association of neurotensin receptors with midbrain dopaminergic cells and with basal forebrain cholinergic cells (Neurobiology of neurotensin, Kitagi and Nemerof eds., Proc. New York Acad. Sciences, 1992). Thus the isolation of neurotensin-receptor expressing cells would make it possible to sort out and selectively administer dopaminergic or cholinergic cells in diseases such as Parkinson's and Alzheimer's disease , respectively. The treatment of these diseases would be greatly improved if the population of cells administered were homogenous, since dissociated embryonic central nervous system preparations may contain cells that may otherwise interfere with the treatment.

40

Embryonic mouse and rat motoneurons have been isolated by Schaffner et al. using flow cytometry (The J. of Neuroscience, 1987, 7:3088-3104). These isolated cells were found to be 80% pure with respect to the presence of fluorescent label in cells examined with fluorescent microscopy immediately after sorting. These results suggest that isolated neurons from human embryonic tissue may be administered to patients in need of brain grafts.

45

It would be also highly desirable to be provided with a marker for neurotensin receptor which allows for receptor physiological studies in tissue slices and in cell cultures and for distinguishing cell surface from intracellular receptor components.

50

SUMMARY OF THE INVENTION

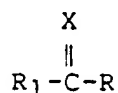
55

One aim of the present invention is to provide for a non-toxic highly sensitive and selective marker of the neurotensin receptor for biochemical, pharmacological and anatomical studies of the said receptor in both brain and peripheral tissues.

Another aim of the present invention is to provide for a marker of neurotensin receptors which would allow for the isolation of neurotensin-receptor expressing cells.

Another aim of the present invention is to provide for a marker of neurotensin receptors which allows for receptor physiological studies *in vivo* or *in vitro* and for distinguishing cell surface from intracellular receptor components.

In accordance with the present invention there is provided a compound having the formula:



or a pharmaceutically acceptable acid salt thereof, wherein

R is a polypeptide moiety comprising an amino acid sequence:

-Y-Arg-Pro-Z-Ile-Leu (SEQ ID NO:1);

wherein

Y is Arg, and

Z is Tyr or Trp;

R₁ is a fluorophore selected from the group consisting of fluorescein, rhodamine, Blue fluorescent and Texas red; and

X is oxygen or sulfur.

In accordance with the present invention there is provided for *in vitro* labeling of neurotensin receptors on cell surface, which comprises the steps of:

- a) incubating dissociated cells, cell culture preparations or tissue slices with a compound of the present invention; and
- b) visualizing said compound of step a) using flow cytometry or epifluorescence/confocal microscopic methods.

In accordance with the present invention there is also provided a method to isolate neurotensin-receptor expressing cells, which comprises the steps of:

- a) incubating dissociated cells or cell culture preparations with a compound of the present invention;
- b) isolating cells bound to the compound of the present invention using flow cytometric cell-sorting methods; and
- c) injecting labeled sorted cells in live animals or in human patients.

In accordance with the present invention there is provided a method for the treatment of Parkinson, Alzheimer and/or others neurodegenerative diseases, which comprises administering to a patient neurotensin-receptor expressing cells isolated according to the method of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the structure of N-fluoresceyl thiocarbamyl-[Glu¹] neurotensin, in accordance with one embodiment of the present invention;

Fig. 2 is an analytical chromatographic profile of a compound of the present invention and of neurotensin;

Fig. 3 is a confocal optical section of a cholinergic/hybrid (SN6) cell incubated with a compound of the present invention;

Fig. 4 is a displacement curve of the binding of a compound of the present invention on mouse brain membranes by iodinated-neurotensin;

Fig. 5 is a light microscopic image of fluoro-neurotensin labeling of dopaminergic neurons in rat brain sections; and

Fig. 6 is a flow cytometric histogram of N-fluorescein isothiocarbamyl-[Glu¹] neurotensin binding on SN6 cholinergic hybrid cells at 4°C in the presence (a) and in the absence (b) of non-fluorescent neurotensin.

DETAILED DESCRIPTION OF THE INVENTION

In the present invention the amino acids are identified by the conventional three-letter abbreviations as indicated below:

5

10

15

20

25

30

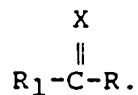
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic Acid	Asp
Cysteine	Cys
Glutamic Acid	Glu
Glycine	Gly
Histidine	His
Leucine	Leu
Lysine	Lys
Methionine	Met
Ornithine	Orn
Phenylalanine	Phe
Proline	Pro
Serine	Ser
Threonine	Thr
Tryptophane	Trp
Tyrosine	Tyr
D-Tyrosine	tyr
Valine	Val

35

The present invention relates to highly sensitive fluorescent probes which allow for rapid and precise characterization of neurotensin receptor binding properties on whole cells.

The fluorescent compounds of the present invention have the following general formula:

40



45

R is a polypeptide moiety which consists essentially of an amino acid sequence selected from the group consisting of:

50

55

	N-term.	C-term.
	-Arg ⁸ -Arg-Pro-Tyr-Ile-Leu ¹³ ;	
	-Arg-Arg-Pro-Trp-Ile-Leu;	
5	-Pro ⁷ -Arg-Arg-Pro-Tyr-Ile-Leu;	
	-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
	-Lys ⁶ -Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
10	-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
	-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
	-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
15	-Asn ⁵ -Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
	-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
	-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
	-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
20	-Glu ⁴ -Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
	-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
	-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
25	-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
	-Tyr ³ -Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
	-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
	-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
30	-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
	-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
	-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
35	-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;	
	-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;	
40		
45		
50		
55		

-Leu²-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 5 -Leu-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -Leu-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 -Leu-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -Leu-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 10 -Leu-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -Leu-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 -Glu¹-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 15 -Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 -Glu-Leu-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -Glu-Leu-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 20 -Glu-Leu-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -Glu-Leu-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 -Glu-Leu-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -Glu-Leu-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 25 -pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 -pGlu-Leu-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 30 -pGlu-Leu-Tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 -pGlu-Leu-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 -pGlu-Leu-tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Trp-Ile-Leu;
 -pGlu-Leu-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Tyr-Ile-Leu;
 35 and
 -pGlu-Leu-tyr-Glu-Asn-Orn-Pro-Arg-Arg-Pro-Trp-Ile-Leu.

40 In accordance with the present invention, each of the amino acid residues identified at positions 1 to 8 may be substituted by Lys or Orn.

Further, the amino acid sequence of the polypeptide moiety in accordance with the present invention may be lengthened at the N- or C-terminal as long as the neurotensin-like biological activity is preserved.

45 In accordance with the present invention, the expression neurotensin-like biological activity is intended to mean that the polypeptide induces biological effects similar to those of neurotensin and/or binds with high affinity and selectivity to the neurotensin receptor.

R₁ is a fluorophore moiety selected from the group consisting of fluorescein, such as fluorescein isothiocyanate, 5-carboxy-fluorescein, 6-carboxy-fluorescein, rhodamine, such as tetramethyl rhodamine isothiocyanate, Blue fluorescent, such as BODIPY™, and Texas red.

50 In accordance with the present invention, other fluorophores may be used where neurotensin-like biological activity is preserved.

In accordance with the present invention, the fluorophores may be linked to the polypeptide moiety at position ranging from 1 to 8 via a thiocarbamyl bond, where X is sulfur, or a peptide bond, where X is oxygen.

55 The preferred compound in accordance with the present invention is N-fluoresceyl thiocarbamyl-[Glu¹] neurotensin (N-FTC-[Glu¹]NT) as shown in Fig. 1.

Although there have been previous attempts at conjugating peptides with fluorescein, N-FTC-[Glu¹]NT is the first example of a successful conjugation of fluorescein with the tridecapeptide neurotensin. The salient features of one compound of the present invention are:

- (1) the selective attachment of the fluorescein molecule to the neurotensin N-terminus;
- (2) the purification of the conjugated compound to approximately 99% purity allowing for optimal detection sensitivity;
- (3) the similarity of its pharmacological properties with those of the native peptide; and
- (4) the fact that it is 100% non-toxic and has a demonstrated shelf life of at least one year.

The fluorescent peptide compounds of the present invention offer a new, inexpensive and highly sensitive tool for biochemical, pharmacological and anatomical studies of the neurotensin receptor in both brain and peripheral tissues. The present fluorescent probes offer several advantages over the use of radioactive compounds.

The compounds of the present invention do not have any of the common drawbacks of radioactive molecules such as short half-life, high cost and slow detection yield (which may imply weeks of photographic exposure). Further, they compensate for two major shortcomings of current neurotensin radioactive probes: their low specific activity (which admittedly is higher with iodinated than tritiated ligands, but also entails greater biohazards) and the fact that they essentially provide static information (i.e. information that is not applicable to studying living processes in real time).

In addition to providing a non-radioactive approach to the characterization of neurotensin receptors, the fluorescent compounds of the present invention may be used for a number of additional applications unsuited to radioactive probes. These include the following:

- (1) these fluorescent compounds may be readily applied to the isolation of neurotensin-receptor expressing cells, using flow cytometric cell-sorting methods. Similarly, receptor binding studies may be carried out on whole cells by flow cytometry.

- (2) The fluorescent compounds of the present invention may be used for real time visualization of physiological processes (receptor aggregation, capping and internalization) using confocal laser microscopy on brain slices or in cell culture preparation. The same technique may be used for distinguishing cell surface with respect to intracellular components.

- (3) Confocal microscopic visualization of the bound fluorescent compounds may be combined with that of other cell markers (e.g. Biocytin™, Lucifer™ yellow) for identification of neurotensin receptors on electrophysiologically recorded cells. It may also be conjugated to the immunocytochemical characterization of the cells and/or compartments harboring the labeled receptors, using appropriate fluorescent-tagged antibodies.

In accordance with one embodiment of the present invention, N-FTC-[Glu¹]NT is prepared according to the following procedure.

1- Fluorescent labeling of neurotensin

[Glu¹]NT was synthesized by solid phase technique using a scheme based on t-Boc chemistry/acid labile amino acid protecting groups. After deprotection of the last N-amino group, acylation was performed by fluorescein isothiocyanate (FITC, Sigma, 6-fold excess) in anhydrous dimethylformamide (DMF) containing 5% N,N-diisopropylethylamine (DIEA) for 2 hours at room temperature with stirring. Completion of the coupling was ascertained by a ninhydrin colorimetric test. The acyl-peptide-resin intermediate was then extensively washed with DMF and dried *in vacuo*. It was submitted to hydrogen fluoride cleavage to deprotect amino acid side chains and to cleave the fluorescein thiocarbamyl (FTC) peptide from the resin. The FTC-peptide was solubilized in trifluoroacetic acid (TFA) and subjected to rotary evaporation *in vacuo*. It was then purified by preparative high pressure liquid chromatography (HPLC) on a parsil 10 ODS-3 Whatman™ column (10-um particle size; 2.2 cm x 50 cm), using a binary solvent system consisting of 0.01% aqueous TFA, pH 2.9 and acetonitrile (CH₃CN)-0.01% TFA and an appropriate gradient. Elution of the peptide was monitored at 214nm. Collected fractions were readily screened by analytical HPLC using both UV and fluorescence detection (excitation, 338 nm; emission, 425 nm), pooled accordingly, evaporated *in vacuo* to remove CH₃CN and lyophilized twice. The purified N-FTC-[Glu¹]NT was analyzed for homogeneity by analytical HPLC on a u Bondapak™ C₁₈ column (10-um particle; 0.39 cm x 15 cm) using appropriate linear gradients of 0.01% aqueous TFA; pH 2.9 and 0.01% TFA-CH₃CN and 0.01M ammonium acetate and CH₃CN (Fig. 3, where the position of [Glu¹]NT and its fluorescent analog FTC-[Glu¹]NT are indicated on the profiles). Its amino acid composition was assessed by quantitative amino acid analysis after acidic hydrolysis *in vacuo* (6N HCl, 110°C, 18h) and carboxypeptidase Y (CPY) digestion (6U/0.3umole, 37°C, 48h).

The site of attachment of the fluorescein derivative molecule to the neurotensin N-terminus was identified as N α -Glu¹. The structure of the fluorescent peptide was confirmed by mass spectral analysis. The degree of homogeneity was determined by U.V. and fluorescence detection to 99%. The modification of semi-protected neurotensin with FITC yielded a selective incorporation of one mole FITC/mole unprotected peptide.

N-FTC-[Glu¹]NT was evaluated to be pure as indicated by a single elution peak from reverse-phase HPLC allowing for optimal detection sensitivity (Fig.2). The molecular weight of N-FTC-[Glu¹]NT is 2080. The compound is freely soluble in distilled water or aqueous buffer, and is stable if protected from light and maintained at 4°C. Finally, FTC-NT is 100% non-toxic.

2-Competitive binding assay

The binding of monoiodo ¹²⁵I-Tyr₃-neurotensin was performed on purified brain membranes from adult male mice as described previously (Sadoul J.L. et al., Biochem. and Biophys. Res. Comm., 1984, 120(1):206-213). Briefly, membranes were incubated with 0.1 nM of radiolabeled peptide in the presence of varying concentrations of N-FTC-[Glu¹]NT in 50 mM Tris HCl pH 7.5 containing 0.2% bovine serum albumin and 1 mM 1,10-phenanthroline. The reaction was carried out for 20 minutes at 22°C and stopped by the addition of 2 ml ice-cold buffer. Membranes were then subjected to immediate filtration over Gelman™-filters (Millipore™) under vacuum using a Millipore™ filtration apparatus. They were then thoroughly washed and their radioactivity content was measured in a gamma counter. The data were expressed as the percentage of specific binding of the radioligand in the absence of competitor. IC₅₀ values were obtained graphically and then corrected for the occupancy by the labeled ligand to obtain K_i values. The K_i values presented are the geometric mean [anti-log of averaged log (K_i) values] +/- SEM. The data were analyzed on an IBM/XT micro-computer using EBDA/LIGAND programs. As can be seen in Figure 4, the fluorescent analogue completely displaces specific ¹²⁵I-Tyr₃-neurotensin binding in a dose dependent manner. Scatchard analysis of the data indicates that the binding is virtually the same as that of native neurotensin with an IC₅₀ of 0.55nM and a pseudo-Hill coefficient of approximately 1.

Example I

In vitro labeling of neurotensin receptors on Rat brain tissue sections

Rats were sacrificed by decapitation, the brains were rapidly removed, blocked in the coronal plane and frozen at -40°C. 25µm-thick frozen sections of the substantia nigra were cut on a cryostat and incubated with 10⁻⁴-10⁻⁶ M fluoro-neurotensin diluted in binding buffer (pH 7.4). The incubations were performed at 4°C in the dark for 60 minutes to allow for equilibration, after which the sections were rapidly rinsed in phosphate-buffered-saline (4 x 60 seconds each), dipped in double-distilled water, and air dried under a cool stream of air. The distribution of the fluorescent labeling was examined under a Leica Diaplan™ microscope using a high pressure 100-W mercury lamp and the appropriate dichroic filter combinations for excitation/emission of fluorescein (485/520 nm).

Controls for these experiments included: (1) examination of sections incubated in the absence of the fluorescent ligand to determine background autofluorescence, and in the presence of a 1000-fold excess of unlabeled ligand for the determination of the non-specific binding; and (2) examination of regions of the nervous system known to be devoid of neurotensin receptors.

The anatomical distribution of specifically bound fluorescent ligand in the ventral midbrain tegmentum is illustrated in Fig. 5.

In the substantia nigra pars compacta the label is seen to be selectively accumulated over nerve cell bodies and proximal dendrites. The labeled neurons are ovoid and fusiform in shape with their long axis oriented parallel to the dorsal surface of the pars reticulata. In the latter, the label is mainly confined to a few scattered perikaria, however several labeled processes are seen to radiate from the cells in the pars compacta.

In the ventral tegmental area, the labeling is intense and associated with both nerve cell bodies and surrounding neuropil. In the parabrachial pigmented division, the labeling is interrupted by areas devoid of label corresponding to the trans-tegmental fiber bundles. The fluorescent labeling was no longer apparent in sections incubated with an excess of unlabeled neurotensin. No autofluorescence was observed except a few orange spots in some cells, typical of lipofuscin aggregates.

Example II

Flow cytometric analysis of fluoro-neurotensin binding to cholinergic hybrids cells

Hybridomas cells (SN6) were produced by the fusion of embryonic septal cells with murine neuroblastoma and generously provided by Hammond et al. (Science, December 1986, 234:1237-1240). SN cells were grown in petri dishes (100 mm²) in Dubelcco's modified Eagle's medium (DMEM) containing 44 mM NaHCO₃ and

10% fetal calf serum (Gibco BRL) in a humidified atmosphere of 90% air, 10% CO₂ at 37°C.

For flow cytometry, the cells (1x10⁶ cell/0.1 ml) were washed in PBS and incubated with various concentrations of fluoro-neurotensin in Hepes-Tris-buffer, pH 7.4, in the presence (non-specific) or the absence (total binding) of a 100-fold excess of unlabeled neurotensin. After incubation at 4°C or 20°C for 1 hour, the cells were washed and analyzed with a Becton-Dickinson™ Facscan flow cytometer and consort 30 software.

Flow cytometric histograms of N-FTC-[Glu¹]NT binding to SN6 cholinergic hybrid cells are illustrated in Fig. 6. The majority (97.4%) of the cells displayed specific fluoro-neurotensin binding at both 4°C and 20°C. Saturation of the binding was both time and temperature dependent. Maximal binding densities were higher at 4°C than at 20°C, presumably reflecting a down regulation of cell surface receptors subsequent to internalization.

Example III

Confocal microscopic visualization of neurotensin binding sites on cholinergic hybrid cells.

SN6 cholinergic hybrid cells were incubated with N-FTC-[Glu¹]NT, under the same conditions as above and the distribution of bound fluorescent molecules was analyzed by confocal microscopy.

Confocal imaging was performed with a Leica™ confocal laser scanning microscope configured with a Leica Diaplan™ inverted microscope (equipped with a 40 x NPL oil immersion fluotar objective of 1.30 numerical aperture), an argon ion laser (488 nm) with an output power of 2-50mW, and a VME bus with an MC 68020/68881™ computer system integrated to an optical disc for image storage. All image generating and processing operations were performed on a Leica™ confocal laser microscope software. Optical scanning images (512 x 512 pixels) of cultured cells were made at 0.1 μm intervals for a total of 26 sections per scanning sequence. From this data volume, a single composite image of each cell was generated using extended focus image construction.

Confocal laser microscopic examination of cells incubated with fluoro-neurotensin at 4°C showed prominent staining of the cell borders, suggesting confinement of the label to the cell membrane. Upon warming of these cells up to 37°C for 10 minutes, the surface fluorescence intensity diminished and multiple small, bright fluorescent particles appeared in the cytoplasm. After 30 min at 37°C, these fluorescent endosome-like elements accumulated and formed a perinuclear ring as illustrated in Fig. 2.

While the invention has been described with particular reference to the illustrated embodiment, it will be understood that numerous modifications thereto will appear to those skilled in the art. Accordingly, the above description and accompanying drawings should be taken as illustrative of the invention and not in a limiting sense.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: Beaudet, Alain
Faure, Marie-Pierre
Gaudreau, Pierrette
- (ii) TITLE OF INVENTION: MARKER FOR NEUROTENSIN RECEPTOR
- 10 (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
15 (A) ADDRESSEE: Cabinet Armengaud Aine
(B) STREET: 3, Avenue Bugeaud
(C) CITY: Paris
(E) COUNTRY: FRANCE
(F) ZIP: 75116
- (v) COMPUTER READABLE FORM:
20 (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
25 (A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
30 (A) APPLICATION NUMBER: CA 2,086,453
(B) FILING DATE: 30-DEC-1992
- (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Peaucelle, Chantal
- 35 (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
40 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (iii) HYPOTHETICAL: NO
- 45 (iv) ANTI-SENSE: NO
- (ix) FEATURE:
(A) NAME/KEY: Modified-site
(B) LOCATION: 4
50 (D) OTHER INFORMATION: /note= ""The exact identity of
position 4 is not yet determined, it is either Tyr
or Trp""
- 55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Arg Arg Pro Xaa Ile Leu
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= Position 1 is Arg, Pro-Arg, A-Pro-Arg, Asn-A-Pro-Arg, Glu-Asn-A-Pro-Arg, B-Glu-Asn-A-Pro-Arg, Leu-B-Glu-Asn-A-Pro-Arg or"

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 4
- (D) OTHER INFORMATION: /note= "The exact identity of Position 4 is not yet determined, it is either Tyr or Trp"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Arg Pro Xaa Ile Leu
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

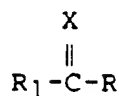
(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

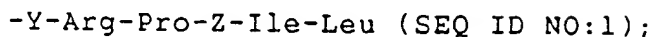
Glu Leu Tyr Glu Asn Lys Pro Arg Arg Pro Tyr Ile Leu
1 5 10

Claims

1. A compound having the formula:



or a pharmaceutically acceptable acid salt thereof, wherein
R is a polypeptide moiety comprises an amino acid sequence:



wherein

Y is Arg, and

Z is Tyr or Trp;

R₁ is a fluorophore selected from the group consisting of fluorescein, rhodamine, Blue fluorescent and Texas red; and

X is oxygen or sulfur.

2. A compound according to claim 1, wherein Y is Arg, Pro-Arg, A-Pro-Arg, Asn-A-Pro-Arg, Glu-Asn-A-Pro-Arg, B-Glu-Asn-A-Pro-Arg, Leu-B-Glu-Asn-A-Pro-Arg or C-Leu-B-Glu-Asn-A-Pro-Arg wherein,

A is Lys or Orn;

B is Tyr or tyr; and

C is Glu or pGlu (SEQ ID NO:2);

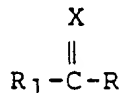
with the proviso that said R₁ is linked to said R via an amino acid residue of Y and that each of said amino acid residue selected from the group consisting of C, Leu, B, Glu, Asn and Pro may be substituted by Lys or Orn.

3. A compound according to claim 1, wherein said polypeptide is neurotensin or functional analogs thereof.
4. A compound according to claim 1, wherein R is Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu, (SEQ ID NO:3)



is fluorescein thiocarbamyl and R is linked via the N-terminus of position 1.

5. A compound having the formula:



or a pharmaceutically acceptable acid salt thereof, wherein

R is an amino acid sequence which comprises at least the amino acid residue 8-13 of neurotensin,

R₁ is a fluorophore selected from the group consisting of fluorescein, rhodamine, Blue fluorescent and Texas red; and

X is oxygen or sulfur.

6. A method for *in vitro* labeling of neurotensin receptors on cell surface, which comprises the steps of:
- incubating dissociated cells, cell culture preparations or tissue slices with a compound according to claim 1; and
 - visualizing said compound of step a) using flow cytometric and/or epifluorescence/confocal microscopic methods.
7. A method according to claim 6, wherein step b) further comprises co-visualization of said compound with other fluorescent markers for histochemical and/or electrophysiological identification of neurotensin re-

ceptor harboring cells.

8. A method to isolate neurotensin-receptor expressing cells, which comprises the steps of:
 - a) incubating dissociated cells or cell culture preparations with a compound according to claim 1;
 - b) isolating cells bound to the compound of claim 1 using flow cytometric cell-sorting methods; and
 - c) injecting labeled sorted cells into live animals or in human patients.
9. A method for the treatment of Parkinson, Alzheimer, and other neurodegenerative diseases, which comprises administering to a patient neurotensin-receptor expressing cells isolated according to the method of claim 8.

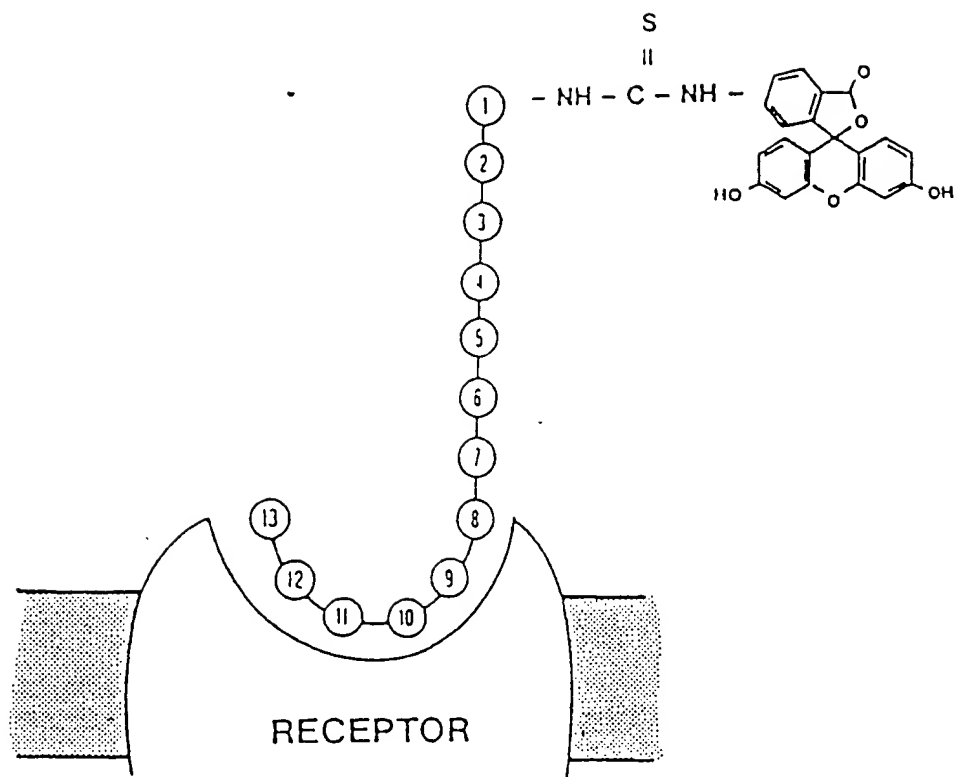


Fig. 1

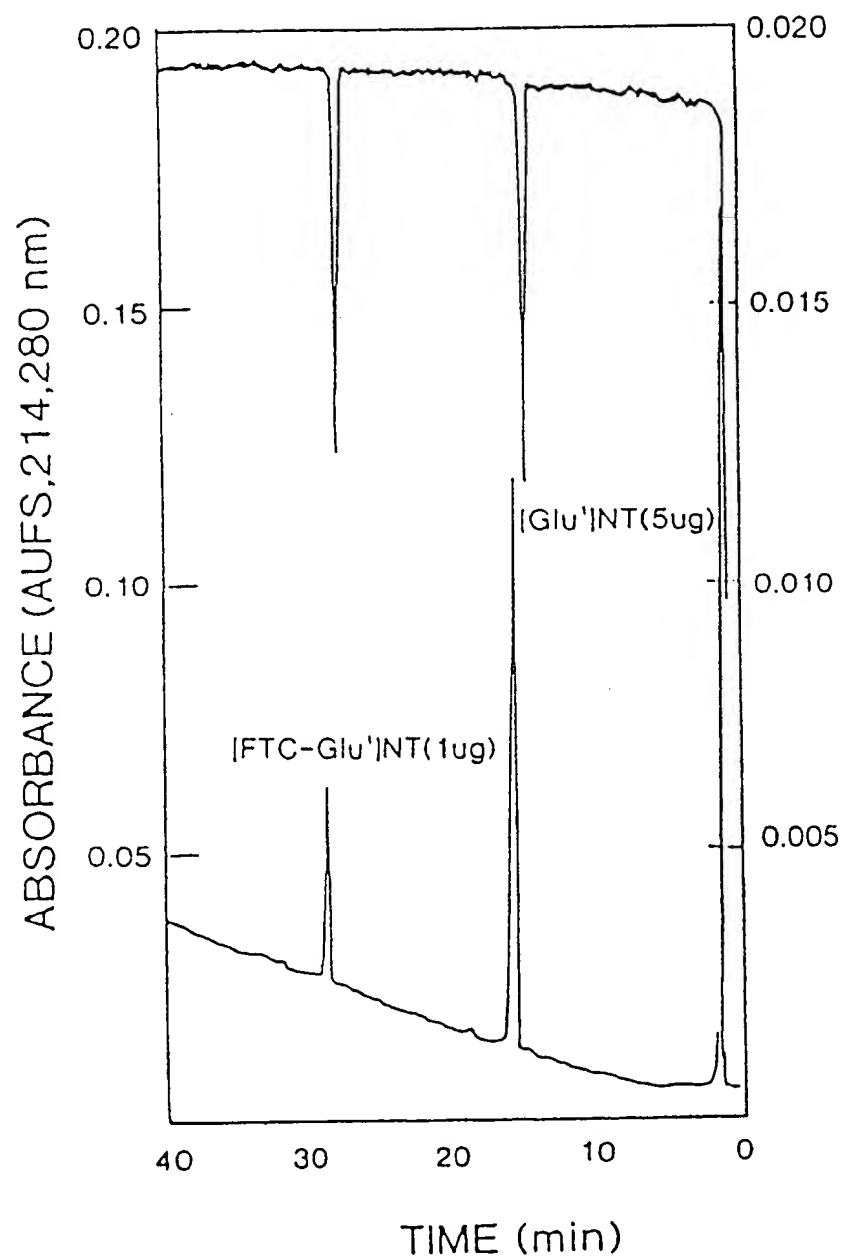


Fig. 2

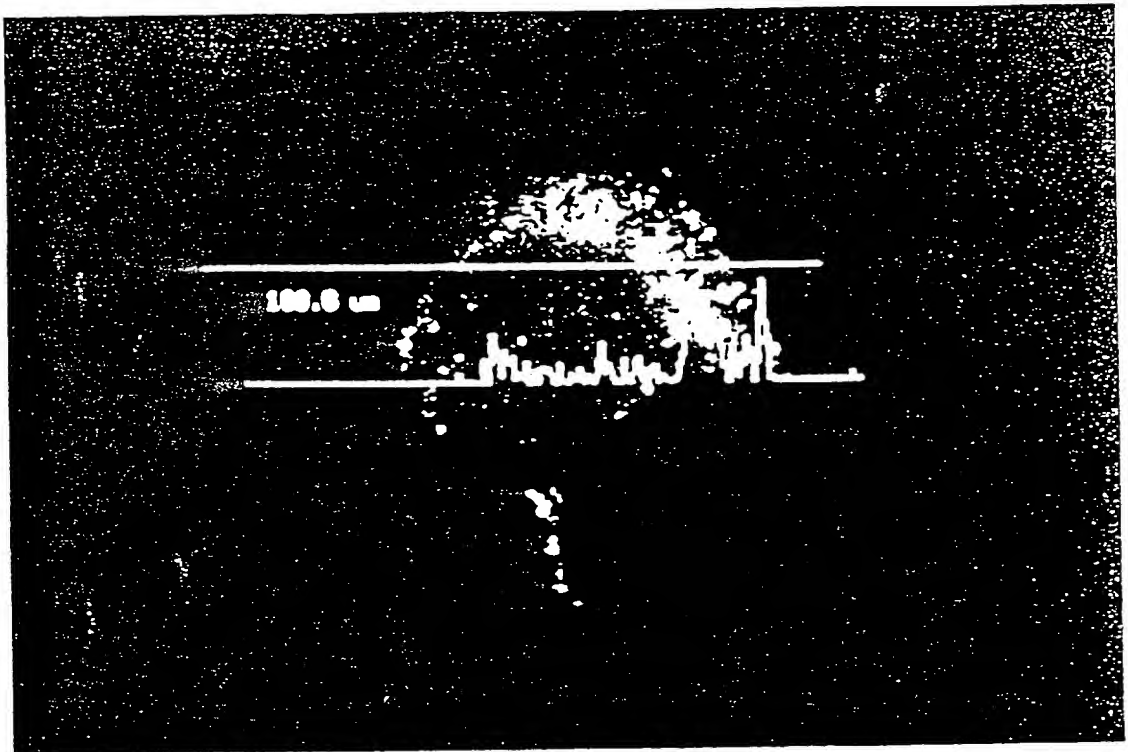


Fig. 3

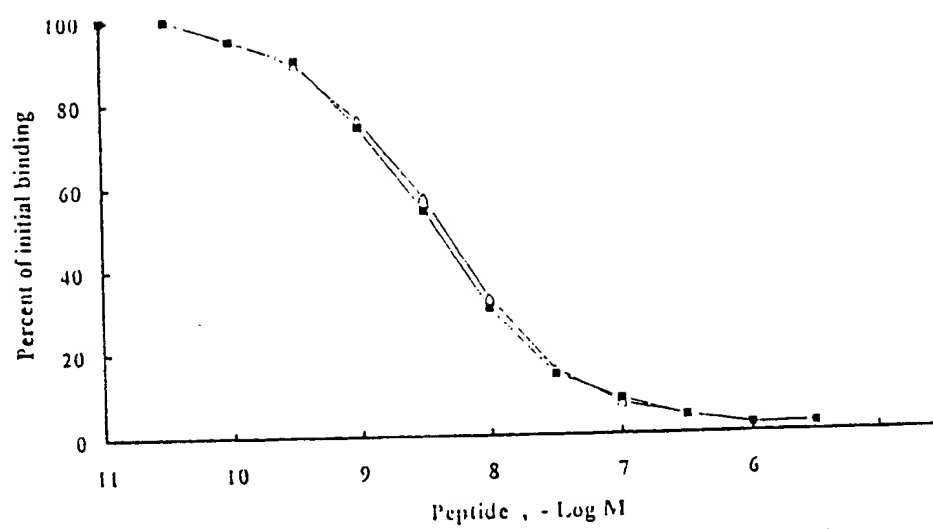


Fig. 4

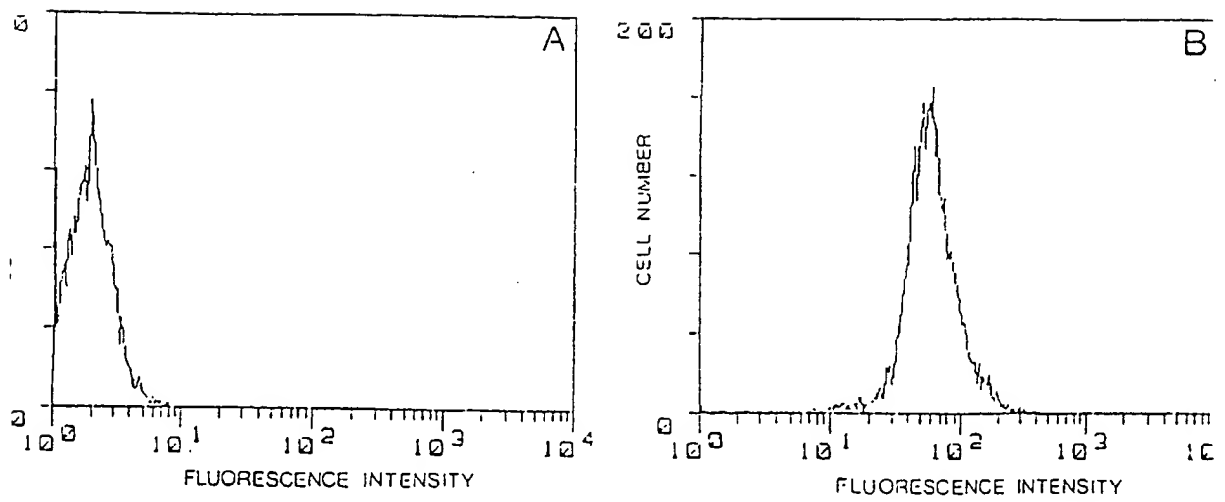


Fig. 5



Fig. 6



Publication number : **0 606 804 A3**

EUROPEAN PATENT APPLICATION

Application number : **93403185.7**

Int. Cl.⁵ : **G01N 33/533, G01N 33/48,
C07K 7/08, G01N 15/14,
G01N 33/68, G01N 33/58**

Date of filing : **27.12.93**

Priority : **30.12.92 CA 2086453**

Date of publication of application :
20.07.94 Bulletin 94/29

Designated Contracting States :
BE CH DE FR GB LI SE

Date of deferred publication of search report :
24.05.95 Bulletin 95/21

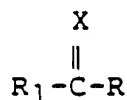
Applicant : **McGILL UNIVERSITY**
845 Sherbrooke Street West
Montreal, Quebec H3A 1B1 (CA)

Inventor : **Beaudet, Alain**
39 Surrey Drive,
Town of Mount Royal
Quebec H3P 1B2 (CA)
Inventor : **Faure, Marie-Pierre**
2286 rue Frenette
Saint-Laurent H4R 1M2 (CA)
Inventor : **Gaudreau, Pierrette**
9123 Rivard Blvd
Brossard, Quebec J4X 1V5 (CA)

Representative : **Peaucelle, Chantal et al**
Cabinet Armengaud Aine
3, avenue Bugeaud
F-75116 Paris (FR)

Marker for neurotensin receptor.

The present invention relates to compound having the formula :



or a pharmaceutically acceptable acid salt thereof, wherein
R is a polypeptide moiety comprises an amino acid sequence :

-Y-Arg-Pro-Z-Ile-Leu (SEQ ID NO:1);

wherein Y is Arg, and

Z is Tyr or Trp ;

R₁ is a fluorophore selected from the group consisting of fluorescein, rhodamine, Blue fluorescent and Texas red ; and

X is oxygen or sulfur. The present invention also relates to a method for the labeling of neurotensin receptors on cell surface and a method to isolate neurotensin-receptor expressing cells.

EP 0 606 804 A3

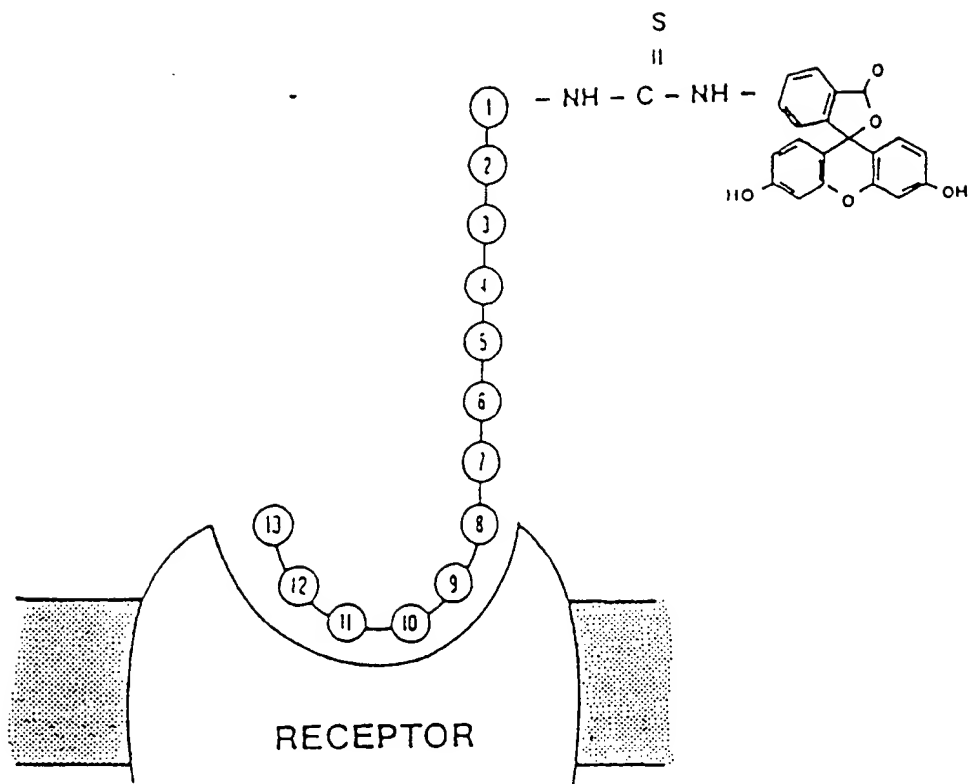


Fig. 1



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention EP 93 40 3185 shall be considered, for the purposes of subsequent proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. CL.5)
A	EP-A-0 333 071 (EISAI CO., LTD.) * page 6 *	1-3,5	G01N33/533 G01N33/48 C07K7/08
D,A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol.248, no.19, 10 October 1973, BALTIMORE, MD pages 6854 - 6861 CARRAWAY ET AL. 'The isolation of a new hypotensive peptide, neurotensin,' * abstract *	3,4	G01N15/14 G01N33/68 G01N33/58
D,A	THE JOURNAL OF NEUROSCIENCE, vol.7, no.10, October 1987, NEW YORK, NY pages 3088 - 3104 SCHAFFNER ET AL. 'Fluorescence-activated cell sorting of embryonic mouse' * abstract *	6,7	
			TECHNICAL FIELDS SEARCHED (Int. CL.5)
			C07K G01N
INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely: 1-7</p> <p>Claims searched incompletely: 8-9</p> <p>Claims not searched:</p> <p>Reason for the limitation of the search:</p> <p>Article 52(4): Method for treatment of the human or animal body by therapy.</p>			
Place of search		Date of completion of the search	Examiner
BERLIN		27 March 1995	Ceder, O
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			

EPO FORM 1503 (03.87) (P04/007)